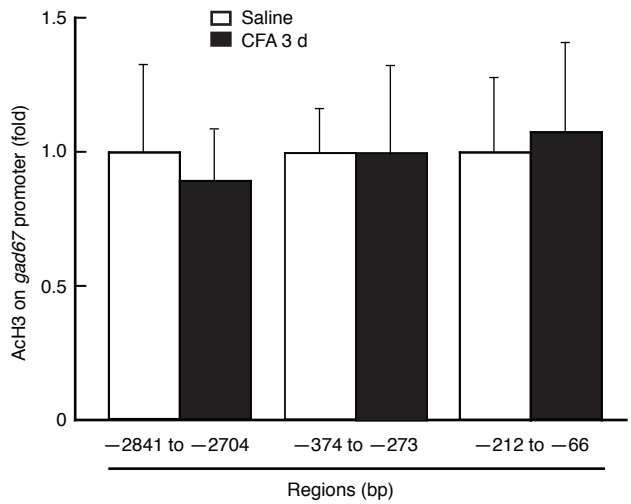
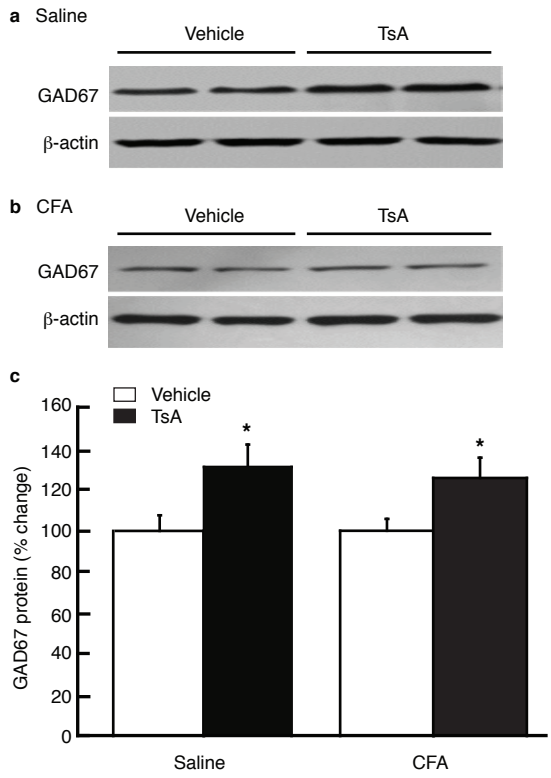
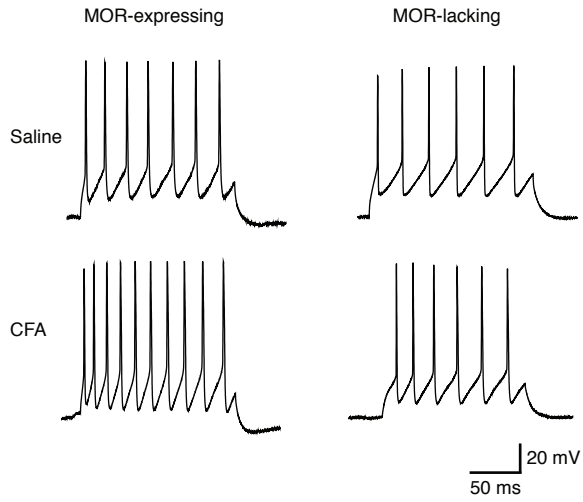
Article Title: Epigenetic suppression of GAD65 expression mediates persistent pain
Authors: Zhi Zhang, Youqing Cai, Fang Zou, Bihua Bie, Zhizhong Z. Pan



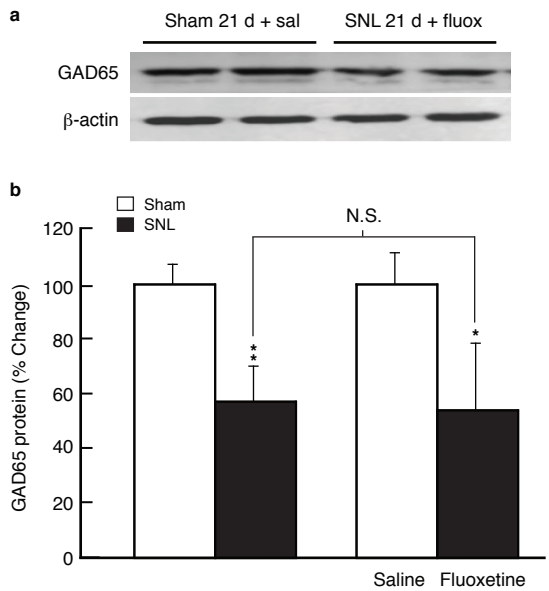
Supplementary figure 1 Persistent pain does not alter acetylated H3 levels in selected promoter regions of *gad67* gene. Normalized levels of acetylated H3 in the indicated sequence regions upstream of the transcription start site in *gad67* gene in NRM tissues from saline- and CFA-injected rats at 3 d post-injection (n = 5 rats each group).



Supplementary figure 2 Histone deacetylase inhibitor increases GAD67 protein expression. (**a**, **b**) Western blots of GAD67 and β-actin proteins in NRM tissues from saline-injected (**a**) and CFA-injected (**b**) rats treated with vehicle or TsA at 3 d post-injection. (**c**) Summarized data of GAD67 protein expression normalized to β-actin in the treatment groups (n = 4–6 rats each group) as in (**a**) and (**b**). * $p<0.05$.



Supplementary figure 3 Persistent pain increases excitability of MOR-expressing NRM cells. Representative firing of evoked action potentials in an MOR-expressing and an MOR-lacking NRM neuron from a saline- and CFA-injected rat.



Supplementary figure 4 An antidepressant drug does not alter neuropathic pain-induced reduction in GAD65 protein expression. (**a**,**b**) Western blots of GAD65 and β-actin proteins (**a**) and group data of GAD65 protein normalized to β-actin (**b**) in saline (sal)- or fluoxetine (fluox)-treated rats after spinal nerve ligation (SNL, $n = 5$ rats) or sham operation ($n = 5$ rats) at 21 d after the surgery. Data were compared with thoseof rats without saline or fluoxetine treatment. N.S., not significant.

SUPPLEMENTARY METHODS

Animals

Male Wistar rats, 9–14 days of age or weighing 200–300 g, were randomly divided into two groups: CFA group and saline control group or SNL and sham groups. The animal colonies were housed on a 12 h light/dark cycle and allowed free access to food and water. *gad65*$^{-/-}$ mice were obtained from the Jackson Laboratories. All procedures involving the use of animals conformed to the guidelines by the University of Texas MD Anderson Cancer Center Animal Care and Use Committee.

Animal models of inflammatory pain and neuropathic pain

To induce hyperalgesia of inflammatory pain, complete Freund's adjuvant (CFA, 40 μl, suspended in a 1:1 oil/saline emulsion, Sigma-Aldrich) or saline was injected into the plantar surface of one hindpaw of a rat or mouse under brief halothane anesthesia and then the animal was returned to home cages. For neonatal rats, a second CFA (20 μl) or saline injection was made on the 3rd day to ensure the persistence of inflammatory pain[20]. To induce mechanical allodynia of neuropathic pain, the left L5 and L6 spinal nerves of a rat under isoflurane anesthesia were surgically isolated and tightly ligated with silk suture, based on the method originally described by Kim & Chung[28]. Pain thresholds were measured every 5 min or daily by the paw-withdrawal test on a freely moving animal with the Hargreaves analgesia apparatus (Stoelting) for thermal hyperalgesia or with von Frey filaments for mechanical allodynia. The maximum possible effect (MPE) = (test latency–baseline latency)/(cutoff–baseline latency). The

cutoff time was 15 s. The antinociceptive effect of an infused drug was measured 10–20 min later.

For systemic treatment of animal models, Trichostatin A (TsA, 4 mg kg^{-1}, dissolved in alcohol as vehicle) or suberoylanilide hydroxamic acid (SAHA, 40 mg kg^{-1}, in DMSO as vehicle) was injected i.p. once daily for 4 days, starting either on the same day (day 0) as CFA injection or 3 d (on day –3) before CFA injection (day 0). NRM tissues were harvested approximately 4 h after the last TsA or SAHA injection. Fluoxetine (10 mg kg^{-1}) was injected i.p. in SNL or sham rats once daily for 21 days.

Extraction of histone proteins and Western blotting

The protocol for histone protein extraction was modified from the one in a previous report[49]. NRM tissues from saline- and CFA-treated rats were homogenized in a hypotonic lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and protease cocktail inhibitors). Lysates were then centrifuged at 16.1 RCF for 10 min at 4 °C. The supernatant was collected, leaving the nuclear pellet for acid extraction (0.2 M HCl). The nuclear pellet and acid buffer were placed overnight and then centrifuged at 16.1 RCF for 10 min at 4 °C. The acid supernatant was collected and ice-cold acetone was added to the pellet and placed on ice for 5 min before centrifuge for 30 min at 4 °C. Finally, the supernatant was removed and remaining histone pellet was placed at room temperature (RT) for 5 min (allowing remaining acetone to evaporate) before being resuspended in 9 M urea. Final protein concentration of histone samples was determined by the Bio-Rad protein assay before immunoblot analysis and 30 μg proteins was mixed with SDS

sample buffer and heated to 100 °C for 5 min. Sample was run on a 12.5% DSD-polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were blocked for 2 h at RT in blocking solution and incubated in histone or acetylated histone antibodies (1:1000, Cell Signaling Technology) and β-actin antibodies (1:1000, Santa Cruz Biotechnology) with agitation overnight at 4 °C. Membranes were then incubated in a 1:5000 dilution of polyclonal secondary antibody to rabbit HRP (Calbiochem) or 1: 20000 dilution of antibody to mouse Ig HRP (Calbiochem) in blocking solution for 1 h at RT. The bands were detected with enhanced chemiluminescence (GE Healthcare) and analyzed with ImageQuant (GE Healthcare) software. The global histone H3 acetylation assays were performed by following the menu of EpiQuik^{TM} Global Histone H3 Acetylation Assay Kit (Epigentek Group Inc). The protocol of Western blotting for total proteins has been described in our previous report[21].

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were modified from the protocol of EpiQuik^{TM} Tissue Acetyl-Histone H3 ChIP Kit (Epigentek Group Inc). Before preparation of cell extraction, antibodies were added to strip wells individually, accompanied by antibody buffer (normal mouse IgG as negative control) and incubated at RT for 60–90 min. We used antibodies to acetyl histone H3 targeting Lys9 and Lys14, acetyl histone H4 targeting Lys12 (Santa Cruz Biotechnology), HDAC1, HDAC2, HDAC4 and HDAC5 (Cell signaling Technology). NRM tissues were harvested and immediately cross-linked in 1% formaldehyde for 15–20 min on a rocking platform at RT. The crosslink reaction was stopped by adding glycine to a final concentration of 0.125 M. The tissue was washed in ice-cold PBS

containing proteinase inhibitors (1 mM PMSF, 1 μg ml^{-1} apoprotin, and 1 μg ml^{-1} pepstatin A) and then frozen on dry ice. The fixed NRM tissue was transferred to Dounce homogenizer and homogenized 10–30 strokes in a cell lysis buffer (10 mM Tris, 10 mM NaCl, and 0.2% NP-40). The homogenate was centrifuged at 5000x g at 4 °C for 5 min and the supernatant was removed. Next, the extracted chromatin was sheared by sonication into 200–500 bp fragments and ten percent of the pre-immunoprecipitated lysate was used as the "input" control for normalization later. The sheared chromatin was diluted 10–fold in ChIP dilution buffer. After removal of the incubated antibody solution, the diluted samples and "input" DNA were transferred to each well for protein/DNA immunoprecipitation at RT for 1 h on a rocking platform. DNA release buffer containing proteinase K was added to the samples (including "input" vial) and incubated at 65 ºC in a water bath for 15 min. Then, reverse buffer was added to the samples and the "input" DNA, and incubated at 65 ºC in a water bath for 90 min to dissociate DNA and histones. After cross-linked DNA reversal, binding buffer was used for DNA precipitation and purification on spin columns with 70% and 90% ethanol. Finally, elution buffer was used to elute purified DNA from the columns. All buffers used above were provided in the EpiQuik™ Tissue Acetyl-Histone H3 ChIP Kit.

Quantification of DNA by real-time PCR

To quantify the level of histone modifications at the gene promoter of interest, quantitative real-time PCR (Applied Biosystems) was used to measure the amount of acetylated, histone-associated DNA. Based on the consensus sequence of cAMP-response element (CRE) for potential binding sites of the transcription factor CREB in the *gad65*

and *gad67* promoter regions, specific primers were designed to amplify representative promoter regions encompassing the CRE sequence from immediately upstream to >2 kb upstream of the TSS. The following primers (Invitrogen) were used: *gad65*, 5'-GCCCTGACTCGAACACTCAC-3' and 5'-ACACAGGGACAGGAAACGTG-3' (-150 to -83 bp); 5'-CTTCCTCCCTCTTTGGTTCCTT-3' and 5'-ACCAGGGAGACCTTGACAATCT-3' (-285 to -153 bp); 5'-ATAAGCAGCAGCCAAGGTCAC-3' and 5'-CGCTGGAGTCTATCACTGAGGA-3' (-646 to -484 bp); and 5'-TCTGCTGCCTCCTTTGTGAA-3' and 5'-CTCCCCACTTCGGATACAGG-3' (-2529 to -2330 bp). *gad67*, 5'-TTGCGCCTCTAGACTTGAGAGT-3' and 5'-TCTCGGAGACAGAAGGGAAAC-3' (-212 to -66 bp); 5'-TGATCTTTTCCCTGCTGTCA-3' and 5'-TCCCATGAGTAATCCAGAACG-3' (-374 to -273 bp); and 5'-AAGAGACAGGCCTGGGATAAAC-3' and 5'-GGTCTGTCTGAGTGATGGGAAG-3' (-2841 to -2704 bp). β-*tubulin*, 5'-TAGAACCTTCCTGCGGTCGT-3' and 5'-TTTTCTTCTGGGCTGGTCTC-3' as controls. Amplifications were run in triplicate by PCR with SYBR® Green PCR Master Mix kit (Applied Biosystems) and each PCR reaction was repeated at least twice independently. The analysis of relative quantification of template was performed as described previously by Tsankova et al[50]. The difference (ΔCt) between control Ct and experimental Ct (CFA) was calculated as: ΔCt = (*NCFA* – *Nave*) x *Ctave*, where *N* is the normalized Ct value of H3 [Ct(H3)/Ct(Input)], *Nave* is the mean *N* value for the control, and *Ctave* is the mean Ct value for control. Fold differences (CFA ChIP relative to control ChIP) were then determined by raising 2 to the ΔCt power. Mean and SEM values

were determined for each fold difference and these values were used in comparison tests adjusted for multiple comparisons to determine statistical significance ($p < 0.05$).

Quantitative RT-PCR

RNA was extracted from rat NRM with the RNAqueous-4PCR Kit (Applied Biosystems) and reverse transcription was performed with the RETROscript Kit (Applied Biosystems). cDNA was quantified by real-time PCR as before[51]. The following primers (Invitrogen) were used to amplify specific cDNA regions of the transcripts: *gad65,* 5'-GCCCAGGCTCATCGCATTCACGTC-3' and 5'-CCTCCACCCCAAGCAGCATCCACA-3'; *gad67*, 5'-GGTTTCTTGCAAAGGACCAA-3' and 5'-CACCAGGGTCACTGTTTTCA-3'; and *gapdh,* 5'-AACGACCCCTTCATTGAC-3' and 5'-TCCACGACATACTCAGCAC-3'. GAPDH quantification was used as an internal control for normalization. Fold differences of mRNA levels over controls were calculated by ΔCt. Each PCR reaction was repeated at least twice independently.

Brain slice preparations and whole-cell recordings

The methods of visualized whole-cell recordings have been described in details previously[21]. Neonatal rats were used in recording experiments due to limited cell visibility and quality in NRM slices from older rats. Both similarities and differences have to be recognized between neonates and adults in the inflammatory responses of NRM neurons for data interpretation[20], and our previous studies have used neonates as a useful and efficient tool with recognized limitations in understanding of cellular

mechanisms of pain behavior in adults[21,29,52,53]. Brainstem slices (200 μm thick) containing the NRM were cut in a vibratome and a single slice was perfused with preheated (35 °C) physiological saline containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2, pH 7.2–7.4. Visualized whole-cell voltage-clamp recordings were obtained from identified NRM neurons with a glass pipette (resistance, 3–5 MΩ) filled with a solution containing the following (in mM): 126 KCl, 10 NaCl, 1 $MgCl_2$, 11 EGTA, 10 HEPES, 2 ATP, and 0.25 GTP, pH adjusted to 7.3 with KOH; osmolarity, 280–290 mOsM. Electrical stimuli of constant current (0.25 ms, 0.2–0.5 mA) were used to evoke GABA-mediated IPSCs. With KCl-filled pipettes and a holding potential of –70 mV, GABA IPSCs were in an inward (downward) direction[21]. GABA IPSCs were recorded in the presence of glutamate receptor antagonists D-(–)-2-amino-5-phosphonopentanoic acid (50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM). A pair of IPSCs was evoked by two stimuli (100 ms apart) and a paired-pulse ratio was calculated by dividing the amplitude of the second IPSC by the first one. Spontaneous miniature IPSCs were recorded in tetrodotoxin (1μM) and analyzed by a sliding IPSC template defined with the acquisition software.

Immunohistochemistry

Frozen coronal sections (20 μm thick) containing the NRM were cut on a cryostat and blocked in PBS containing 0.1% Triton X-100 (Fisher) plus 5% normal donkey serum (Jackson ImmunoResearch Laboratories) for 1 h at RT. Sections were incubated overnight at 4 °C in 1% bovine serum and 0.3% Triton X-100 in PBS with primary

antibodies to synapsin I (1:200, Synaptic Systems) and GAD65 (1:1000, Millipore). Sections were then rinsed in PBS and incubated for 1 h at RT with a mixture of FITC- (1:1000, Jackson ImmunoResearch Laboratories) and Cy3-conjuaged secondary antibodies (1:1000, Jackson ImmunoResearch Laboratories). After additional PBS rinse, sections were mounted on slides using ProLong Gold antifade reagent with DAPI (Invitrogen). A single optimized acquisition exposure time was used for all images acquired from a particular slide. Signals of immunohistochemical staining for GAD65 and synapsin I, and their overlap among different experimental groups (n = 5–6 rats each group) were obtained from randomly selected sections (n = 4–6 sections from each rat) and quantitatively compared manually with the experimenter blind to treatment groups.

Microinjection

Detailed methods of repeated NRM infusions and behavioral pain tests were the same as previously reported[21,53]. A 26-gauge double-guide cannula (Plastics One) was implanted into the brain of an anesthetized rat, aiming the NRM (anteroposterior, –10.0 from the Bregma; lateral, 0; dorsoventral, –10.5 from the dura)[54]. After recovery from the implantation surgery for >5 days, the rat received an intraplantar injection of CFA or saline. NRM infusions were made through a 33-gauge double-injector with an infusion pump at a rate of 0.2 μl min^{-1}. TsA (16.5 mM in 1 μl) or SAHA (100 μM in 1 μl) was infused into the NRM once daily for 4 days on the same schedule as that for the systemic treatment. As a standard control, TsA infusions into a site 1 mm dorsal to the NRM were without effect (data not shown). For mice, TsA was similarly infused into the NRM (anteroposterior, –5.75 from the Bregma; lateral, 0; dorsoventral, –5.5 from the dura).

Statistical analyses and materials

ANOVA (one-way and two-way) and post hoc analysis were used to statistically analyze experimental data between treatment groups with multiple comparisons. Simple comparisons of data were made with the Students' t tests. Data are presented as mean ± SEM and $p < 0.05$ was considered statistically significant. Drugs were purchased from Sigma or Tocris Cookson except SAHA (Cayman Chemical).